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Enzyme Activity and Composition of Myelin and Subcellular Fractions in the Developing Rat Brain

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1. Subcellular fractions and myelin were isolated from developing and adult rat brain. 2. Measurements of chemical composition and enzyme activities indicate the presence of a second myelin-like fraction mainly in the brain of developing rats. 3. This membrane fraction has a different lipid composition from myelin, but resembles myelin in its content of phosphohydrolase and aminopeptidase activity. 4. It is suggested that the second myelin-like fraction may be a submicrosomal contaminant or it may be derived from oligodendroglial plasma membrane during myelinogenesis.

Although myelin isolated from the brains of mature animals has a remarkably constant composition, marked changes during development have been noted in the lipid composition of fractions isolated by centrifugation of the brains of developing rodents (Cuzner & Davison, 1968; Eng & Noble, 1968; Horrocks, 1968; W. T. Norton, unpublished work). The lipid composition of this so-called 'early' myelin resembles that of cellular membranes rather than mature myelin, for it contains less cerebroside and more phospholipid than is found in the myelin from adult brain. It has been proposed (Davison, Cuzner, Banik & Oxberry, 1966; Banik, Blunt & Davison, 1968) that 'early' myelin fractions contain adult-type myelin and a pro-myelin fraction that is derived from the oligodendroglial plasma membrane. The presence of both mature myelin and a second membrane fraction has been found in crude myelin isolated from developing rat brain (W. T. Norton, A. N. Davison & M. Spohn, unpublished work), but relatively little of the second fraction can be obtained from mature brain.

Since it is possible that this second membrane fraction is a contaminant derived from other subcellular structures, in the present paper we describe further work on the composition and enzyme activity of the fraction in comparison with that of other brain subcellular structures. In order to define the components of 'crude' myelin in the developing brain more precisely, special attention has been paid to the distribution of marker enzymes and the analysis of the phosphoglyceride fatty acid spectrum of the myelin-like fraction.

EXPERIMENTAL

Materials. White Wistar rats of either sex were used throughout this work. Animals up to 15 days old were decapitated and older rats were lightly anaesthetized with chloroform before exsanguination. Brains were quickly removed into precooled beakers, weighed and stored in ice.

Preparation of subcellular fractions. This was carried out by the method of Cuzner & Davison (1968) except for the preparation of the microsomal fraction. The supernatant obtained after sedimentation at 13500*g* was recentrifuged in a MSE Superspeed 50 centrifuge with the 8 × 25ml. angle-head no. 2408 rotor, at 50000*g* for 1hr. at 2°. The resulting pellet was defined as the microsomal fraction. Myelin, nerve-ending particles and mitochondrial fractions were prepared by the method described in detail by Cuzner & Davison (1968). The mitochondrial pellet was suspended in 0·32M-sucrose solution and homogenized in a loose-fitting Teflon homogenizer (three or four strokes by hand) with an equal volume of 1·2M-sucrose solution to give a final 0·8M-sucrose solution. A 6ml. portion of the 0·8M-sucrose solution was layered over 6ml. of 1·2M-sucrose solution and overlaid with 6ml. of 0·32M-sucrose solution (Cuzner & Davison, 1968). The upper crude myelin layer was diluted 15–20-fold with ice-cold water and left in ice for 30min. The tubes were centrifuged at 40000*g* for 1hr. at 2°. The pellet was suspended in 0·32M-sucrose and homogenized by hand in a loose-fitting Teflon homogenizer and layered over an equal volume of 0·85M-sucrose solution. The tubes were centrifuged at 53000*g* for 1hr. at 2°, and myelin was collected as a white layer at the interface. A cream-coloured pellet formed at the bottom of the tube was designated the second myelin-like fraction.

Electron microscopy. Samples were fixed in buffered osmium tetroxide solution, pH7·3 (Palade, 1952), and washed several times in 0·1M Sorensen's phosphate buffer, pH7·4. Specimens were stained in 1% (w/v) phosphotungstic acid in ethanol.

Chemicals. ATP (disodium salt), AMP (disodium salt), 2′-AMP, *o*-nitrophenyl acetate, acetylthiocholine iodide, butyrylthiocholine iodide, 5,5′-dithiobis-(2-nitrobenzoic acid), β-naphthylamine, Triton X-100, diazo-2-amino-4-chloroanisole (Diazo Red RC), *o*-nitrophenol, L-leucyl-2-naphthylamide and fatty acid standards were all obtained from Sigma (London) Chemical Co. Ltd., London S.W.6; 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride and 2′,3′-(cyclic)-AMP (sodium salt) were obtained from British Drug Houses Ltd., Poole, Dorset.

Assay of enzyme activities. Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) and cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) were assayed by the method of Ellman, Courtney, Andres & Featherstone (1961). The Unicam SP. 800 recording spectrophotometer was used for the assay at room temperature.

$Na^+ + K^+ + Mg^{2+}$-effected ATPase* (ATP phosphohydrolase, EC 3.6.1.3) was assayed by the method of Schwartz, Bachelard & McIlwain (1962). The final concentrations of Na^+ and K^+ in the incubation mixture were 100mM and 30mM respectively. The reaction was carried out at 37° for 10min. after 5min. preincubation. The P_i released was determined from a portion of the supernatant by the method of Martin & Doty (1949). Enzyme activity is expressed as μmoles of P_i liberated/hr./g. wet wt. of tissue.

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity was assayed by the method described by Emmelot, Bos, Benedetti & Rümke (1964). P_i was determined by the same method as described for ATPase. Enzyme activity is expressed as μmoles of P_i liberated/hr./g. wet wt. of tissue.

Succinate dehydrogenase [succinate–(acceptor) oxidoreductase, EC 1.3.99.1] activity was assayed by the method of Laatsch, Kies, Gordon & Alvord (1962). Here the 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride concentration used was 10mg./ml. as was stated in the original method.

Aryl esterase (aryl ester hydrolase, EC 3.1.1.2) activity with *o*-nitrophenyl acetate as substrate was assayed by the method of Sellinger & De Balbian Verster (1962). Enzyme activity is expressed as μmoles of *o*-nitrophenol liberated/hr./g. wet wt. of tissue.

Leucine aminopeptidase (L-leucyl-peptide hydrolase, EC 3.4.1.1) activity was assayed by the Bratton–Marshall method as modified by Adams & Glenner (1962).

2′,3′-Cyclic nucleotide 3′-phosphohydrolase activity was assayed by the method of Drummond, Iyer & Keith (1962), as modified by Kurihara & Tsukada (1967). Tubes were preincubated for 3min. before final incubation. All the samples were sonicated for 10min. at 5A (Soniprobe type Lii30A; Dawe Instruments Ltd., London W.3). After centrifugation a sample (0·03ml.) of supernatant was spotted on Whatman no. 1 chromatography paper. The chromatograms were developed in propan-2-ol–30% (w/w) ammonia–water (7:1:2, by vol.) (Markham & Smith, 1952) for 4hr. The chromatogram was dried and the spot of 2′-AMP was located under u.v. light and marked. The spot was cut out and eluted with 4ml. of 0·01M-HCl. The extinction of the eluted solution was read at 260nm. in a Unicam SP. 800 spectrophotometer. The enzyme activity was calculated from the 60% hydrolysis of the substrate (Drummond *et al.* 1962) and expressed as μmoles of 2′-AMP formed from 2′,3′-(cyclic)-AMP/hr./g. under the conditions described above.

Determination of protein. A modification of the biuret method of Gornall, Bardawill & David (1949) was used for determination of protein. This method gave similar results to that of Lowry, Rosebrough, Farr & Randall (1951). Crystalline bovine serum albumin (fraction V; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) was used as standard.

Determination of nucleic acids. Total nucleic acid content of the tissue was determined by the Schneider (1945) method as described by McIlwain & Rodnight (1962) except that the trichloroacetic acid-precipitable residue left after washing with 95% ethanol was rewashed with 2ml. of chloroform–methanol–36% (w/w) HCl (200:100:1, by vol.) to eliminate polyphosphoinositides. The residue was then heated at 90° for 15min. in a water bath with 5ml. of 5% (w/v) trichloroacetic acid. After cooling and centrifuging, the extinction of the supernatant was determined at 260nm. in a Unicam SP. 800 spectrophotometer.

Determination of fatty acids. Fatty acids were isolated from total lipid extract and methylated as described by Rathbone (1965). The methyl esters of fatty acids were separated by g.l.c. (Perkin–Elmer model F11 instrument). The stationary phase used was diethylene glycol succinate on hexamethyldisilazane–Chromosorb W (80–100 mesh) packed in a stainless-steel column, 2m. long × $\frac{1}{4}$in. outer diam. The column was run at 190°, with N_2, H_2 and air at 20, 15 and 30lb./in.^{2} respectively. The major fatty acids were identified by using standard samples; other fatty acid esters were identified from log(retention time) curves (James & Martin, 1956; Woodford & Vangent, 1960). The fatty acid content (C_{14} to C_{20}) was calculated by triangulation as described by Biran & Bartley (1961). Other fatty acids ($C_{12:0}$, $C_{12:1}$, $C_{13:0}$, $C_{13:1}$, $C_{14:1}$, C_{15}, $C_{20:0}$, $C_{22:2}$, $C_{22:5}$, and $C_{24:0}$) were present in very small amounts and they were not measured.

RESULTS

Subcellular fractionation. Fractionation of developing and adult rat brains was undertaken on homogenates prepared in 0·32M-sucrose with slight modification of the conditions described by Cuzner & Davison (1968). Samples were checked by electron microscopy to ensure morphological control of the separative procedures. Protein and total nucleic acid content of each fraction was always measured (Table 1) and the results are compared wherever possible with those in the literature. Relatively small differences were noted in the content of total nucleic acid in each subcellular fraction with increasing age. Only relatively small increases in protein content of mitochondria and nerve-ending particles were seen (see Abdel-Latif, Brody & Ramahi, 1967; Rubiolo De Maccioni & Caputto, 1968; Gregson & Williams, 1969). The only marked change observed was a sixfold increase in total crude myelin protein isolated from the adult rat brain compared with that found in the 12-day-old brain. This dramatic increase in myelin is in conformity with the observations of others (e.g. W. T. Norton, unpublished work).

To check the homogeneity of different subcellular fractions we examined the distribution of certain marker enzymes in each fraction. The enzyme activity is expressed as μmoles of substrate utilized/hr./g. wet wt. and as a percentage recovery of the total homogenate (Tables 2 and 3). Tables 2 and 3 also indicate the suggested cellular and subcellular localization ascribed to each enzyme. The distribu-

* Abbreviation: ATPase, adenosine triphosphatase.

Table 1. *Distribution of protein and total nucleic acid in subcellular fractions of developing rat brain*

Methods of determining protein and nucleic acid were described in the text. Results are expressed as mg./g. wet wt. tissue. Numbers in parentheses indicate number of separate experiments. Each fractionation was from at least four rats (12–15 days old) and at least two for 21-day-old and adult rats.

	Protein (mg./g. wet wt. of brain)				Nucleic acid (mg./g. wet wt. of brain)			
Age (days)	12 (5)	15 (5)	21 (3)	Adult (5)	12 (3)	15 (2)	21 (2)	Adult (2)
Brain wet wt. (g.) ...	0·987±0·08	1·22±0·05	1·41±0·11	1·84±0·16				
Body wt. (g.) ...	14·97±1·73	27·58±2·10	43·50±3·05	200–250±10·40				
Fractions								
Whole homogenate ...	67·9±1·46	82·6±5·07	91·5±3·9	111·5±4·20	3·05±0·10	2·90	2·40	2·25
Microsomes	11·2±0·31	13·4±2·17	15·5±1·67	11·9±0·81	0·64±0·02	0·63	0·44	0·40
Crude mitochondria ...	—	41·7±2·82	49·6±2·30	62·3±4·10	—	—	—	—
Purified mitochondria	5·3±1·18	7·0±1·30	10·3±2·15	8·3±0·02	0·27±0·01	0·22	0·26	0·13
Nerve-ending particles	16·4±1·49	22·3±2·92	22·6±2·30	29·0±1·1	0·19±0·01	0·21	0·10	0·13
'Crude' myelin ...	1·8±0·01	5·15±0·22	8·2±0·15	13·4±0·02	0·02±0·003	0·04	0·05	0·01
Nuclei	12·2±1·67	12·2±1·09	11·1±2·2	19·1±1·76	1·23±0·01	1·23	1·06	1·01
Supernatant ...	20·4±1·78	23·5±1·58	21·3±2·50	20·9±2·23	0·56±0·02	0·32	0·30	0·24

tion of three enzymes whose activity is typically localized in microsomal membranes was investigated. The activities of total $Na^+ + K^+ + Mg^{2+}$-effected ATPase (see Abdel-Latif *et al.* 1967), acetylcholinesterase and aryl esterase in the whole homogenate of the brain of 21-day-old rats was close to that of the adult brain, whereas in the 12-day-old animal the activities were about half. Similar changes were seen in enzyme distribution in each subcellular fraction; the threefold increase with age in acetylcholinesterase activity in nerve-ending particles is noteworthy. Although the results in Table 2 show increases in total enzyme activity in all subcellular fractions as a function of increasing age, when results are calculated as specific activities (μmoles of substrate/hr./mg. of protein) the changes are less marked (see Table 7). Cholinesterase is claimed to be present in large amounts in glial cells (Giacobini, 1962) and our results (Tables 2 and 3) show an increase in the enzyme activity of the whole brain that is not inconsistent with the rate of multiplication of glial cells within the rat central nervous system (Schonbach, Hu & Friede, 1969). Less change in the total enzyme activity was seen with development in the crude myelin fraction compared with that of the nerve-ending particles, but again when the results are calculated as specific activities these differences become negligible. Cholinesterase activity was highest in the adult nuclear (23% of activity of whole homogenate) and microsomal fractions (22%), confirming earlier work of Aldridge & Johnson (1959). Total activity of ATPase, acetylcholinesterase and arylesterase was highest in the nerve-ending fraction (16–50% of that of the whole homogenate), but on the basis of specific activity the microsomal fraction had the highest enzyme activity. As expected, as much as 85% of succinate dehydrogenase activity is localized in the crude mitochondrial pellet, two to three times as much activity being found in free mitochondria as in the nerve-ending-particle fraction (De Robertis, Pellegrino De Iraldi, De Lores Arnaiz & Salganicoff, 1962; Whittaker, Michaelson & Kirkland, 1964; Balázs, Dahl & Harwood, 1966; Salway, Kai & Hawthorne, 1967). During development from 12 days onwards the proportion of succinate dehydrogenase in the nerve-ending-particle fraction decreases and there is a notable increase in the specific activity of this enzyme in the free purified mitochondria. The distribution of two enzymes, leucine aminopeptidase and 2′,3′-cyclic nucleotide 3′-phosphohydrolase thought to be typically localized in the myelin fraction was also studied (Table 3). During the period of myelination there was little increase in the activity of leucine aminopeptidase/g. of whole brain and no increase was noted in the enzyme found in myelin. In contrast the activity of the 2′,3′-cyclic nucleotide 3′-phosphohydrolase in the whole brain increases with myelination and especially marked changes are seen in the myelin fraction. The distribution of 5′-nucleotidase was also studied, since this is claimed to be a marker enzyme for the plasma membrane (Coleman & Finean, 1966; Song, Rubin, Rifkinda & Kappos, 1969). A significant increase in enzyme activity was seen during development, but no change was seen in the distribution of the enzyme at the ages we examined.

Myelin subfractions in the developing brain. Purified myelin and the secondary myelin-like fraction were separated from developing brain as described above. The purified myelin was found by electron microscopy to have the appearance of adult myelin with the same periodicity. The second fraction had the appearance of single membrane vesicles and resembled some brain microsomal subfractions

Table 2. *Subcellular distribution of marker enzymes in developing rat brain*

Fractions were isolated by differential centrifugation as described by Cuzner & Davison (1968). Enzyme activity was measured by methods described in the text. The results are the average of several determinations, the numbers being shown in parentheses. Standard deviations are recorded where more than two experiments were carried out.

References: [1] Aldridge & Johnson (1959); [2] Albers, De Lores Arnaiz & De Robertis (1965); [3] Stahl (1968); [4] De Robertis *et al.* (1962); [5] Giacobini (1962).

Reported localization	Enzymes	Distribution	Activity (μmoles of substrate/hr./g. wet wt.				Distribution (%)			
		Age (days)	12	15	21	Adult	12	15	21	Adult
External synaptic membranes, microsomes[2,3]	$Na^+ + K^+ + Mg^{2+}$-effected ATPase	Whole homogenate	686·30 (2)	1528±56 (4)	2047 (2)	2593±38·9 (3)	100	100	100	100
		Microsomes	119·35	233±9	302	401±11	17·41	15·24	14·75	15·46
		Crude mitochondria	—	1016±89	1230	1485±42	—	66·49	60·08	57·26
		Nerve-ending particles	191·40	425±43	660	864±53	27·90	27·81	32·24	33·32
		Purified mitochondria	81·90	170±37	177	267±27	11·91	11·12	8·64	10·29
		Crude myelin	34·20	53±11	85	127±9·2	4·98	3·46	4·15	4·89
		Nuclei	75·41	97±7	182	320±10·2	10·98	6·34	8·89	12·34
		Supernatant	92·30	104±12	114	160±20	13·40	6·80	5·56	6·17
External synaptic membranes, microsomes[4,1]	Acetylcholinesterase	Whole homogenate	223·4 (3)	296·9±39·23 (3)	408·6±30·26 (3)	582±39·23 (3)	100	100	100	100
		Microsomes	43·0±9·2	76·5±13·51	101·6±10·20	123·2±13·60	19·24	25·76	24·85	21·16
		Crude mitochondria	—	168±21·37	—	360±33·68	—	66·58	—	61·85
		Nerve-ending particles	79·4±5·18	116·3±12·60	155·4±11·93	258·5±21·07	35·54	39·17	38·03	44·41
		Purified mitochondria	12·0±1·33	12·2±0·19	15·0±1·03	15·7±1·31	5·37	4·10	3·67	2·69
		Crude myelin	4·4±0·95	8·9±0·31	22·6±3·42	33·4±5·3	1·96	2·99	5·53	5·73
		Nuclei	15·6±1·76	29·6±2·31	21·0±0·98	21·0±1·01	6·98	9·96	5·13	3·60
		Supernatant	50·6±8·11	57·6±5·65	91·8±9·34	106·2±6·61	22·64	19·40	22·46	18·24
Glial cells[5]	Cholinesterase	Whole homogenate	13·7±1·13 (3)	17·2±1·47 (3)	25·8±2·31 (3)	40·95±9·40 (3)	100	100	100	100
		Microsomes *	3·9±0·25	3·8±0·13	4·3±0·24	8·90±1·62	28·46	22·09	16·66	21·73
		Nerve-ending particles	1·8±0·09	3·0±1·05	2·4±0·06	8·0±1·37	13·13	17·44	9·30	19·53
		Purified mitochondria	1·9±0·13	1·7±0·06	2·1±0·04	3·3±0·43	13·86	9·88	8·13	8·05
		Crude myelin	0·72±0·03	1·0±0·05	1·1±0·03	2·3±0·07	5·26	5·81	4·26	5·61
		Nuclei	2·8±0·18	3·4±0·21	5·0±0·31	9·7±1·70	20·43	19·76	19·37	23·68
		Supernatant	3·9±0·10	5·6±0·41	6·8±0·28	7·8±0·36	28·46	32·55	26·36	19·04

Microsomes[1]	Aryl esterase	Whole homogenate	225·0±11·43 (3)	280·0±34·2 (3)	414·7±41·82 (3)	500·0±38·53 (3)	100	100	100	100
		Microsomes *	67·8±5·27	77·5±8·4	107·9±10·39	112·5±9·83	30·13	27·67	26·01	22·50
		Nerve-ending particles	42·0±3·96	51·0±4·1	80·0±7·25	96·0±8·15	18·66	18·21	19·29	19·20
		Purified mitochondria	19·5±1·82	22·0±2·2	32·5±2·50	30·0±1·43	8·66	7·85	7·83	6·00
		Crude myelin	4·6±1·21	14·0±2·6	17·1±3·62	26·5±2·62	2·04	5·00	4·12	5·30
		Nuclei	17·8±1·48	26·0±2·0	32·0±1·93	24·0±1·52	7·91	9·28	7·71	4·80
		Supernatant	58·5±5·87	104·0±6·1	104·0±3·67	163·9±12·14	26·00	37·14	25·07	32·78

* Crude mitochondrial cholinesterase and aryl esterase activity not assayed.

(Sellinger, De Balbian Verster, Sullivan & Lamar, 1966) or fractions of isolated synaptic membranes (see, e.g., De Robertis *et al.* 1962). It was confirmed that the purified myelin from developing rat brain had a similar composition to that of the adult brain, but that the second myelin-like fraction was deficient in cerebroside and had relatively more phospholipid (Table 4). The phosphoglyceride fatty acid pattern of purified myelin from a 15-day-old rat and from an adult differed in chain length of fatty acid (see also Marshall, Fumagalli, Niemiro & Paoletti, 1966). In the myelin from the developing rat much more $C_{16:0}$ fatty acid esters were present than in the adult (Table 5 and 6), although more $C_{18:1}$ acid was found in the adult than in developing brain (Banik & Davison, 1967). The second myelin-like fraction had a higher proportion of short-chain fatty acid esters (C_{16} and below) in comparison with adult rat myelin (Tables 6) and thus resembled the fatty acid pattern of other cellular membranes (Biran & Bartley, 1961). The enzyme profiles of crude and purified myelin and the second myelin-like fraction are shown in Table 7, where results are recorded as specific activities. Comparison shows that both the myelin and the second myelin-like fraction have very little succinate dehydrogenase activity and neither fraction can be regarded as derived from inner membranes of the mitochondria. In the second myelin-like fractions from a 15-day-old rat the specific activities of ATPase and of acetylcholinesterase are respectively one-half and one-quarter that of microsomal and nerve-ending-particle fractions. The ATPase and acetylcholinesterase specific activities in the adult second myelin-like fraction are, however, closer to those of the microsomal and nerve-ending-particle fractions respectively. In contrast with the other enzymes studied, the specific activity of 2′,3′-cyclic nucleotide 3′-phosphohydrolase is higher in both myelin and the myelin-like fraction compared with any other fraction in 15-day-old and adult brain.

DISCUSSION

Development of the central nervous system occurs in three broad stages (Davison & Dobbing, 1968). In the rat brain early establishment of cell populations phases into growth of dendrites and axons. This latter stage overlaps the commencement of myelination, which in the rat begins at about 10 days after birth. The adult concentration of synaptic endings and mitochondria (expressed as mg. dry wt./g. of wet cerebral tissue) is probably reached by about 25 days after birth (Rubiolo De Maccioni & Caputto, 1968), whereas accumulation of myelin continues at an exponential rate for at least a year (W. T. Norton, unpublished work).

Table 3. *Subcellular distribution of marker enzymes in developing rat brain*

Fractions were isolated by differential centrifugation as described by Cuzner & Davison (1968). Enzyme activity was measured by methods described in the text. The results are the average of several determinations, the numbers being shown in parentheses. Standard deviations are recorded where more than two experiments were carried out.

References: [1] Aldridge & Johnson (1959); [2] De Robertis *et al.* (1962); [3] Emmelot *et al.* (1964); [4] Coleman & Finean (1966); [5] Adams, Davison & Gregson (1963); [6] Beck, Hasinoff & Smith (1968); [7] Kurihara & Tsukada (1967).

Reported localization	Enzyme	Distribution	Activity (μmoles of substrate/hr./g. wet wt.)				Distribution (%)			
		Age (days) ...	12	15	21	Adult	12	15	21	Adult
Mitochondria[1,2]	Succinate dehydrogenase	Whole homogenate	186·13 ± 12·50 (3)	329·20 ± 10·70 (4)	438·93 ± 71·60 (3)	481·30 ± 68·10 (3)	100	100	100	100
		Microsomes	12·70 ± 1·79	19·43 ± 2·72	17·53 ± 1·83	22·53 ± 1·67	6·82	5·91	3·92	4·68
		Crude mitochondria	—	245·80 ± 15·73	352·70 ± 21·30	412·60 ± 62·50		74·50	80·50	86·00
		Nerve-ending particles	96·20 ± 2·86	156·03 ± 11·91	153·37 ± 3·30	95·86 ± 7·55	51·52	47·48	35·00	19·80
		Purified mitochondria	53·75 ± 5·0	83·33 ± 2·90	185·93 ± 13·31	301·00 ± 43·09	28·89	25·30	42·40	62·49
		Crude myelin	1·63 ± 0·03	2·64 ± 1·09	2·70 ± 0·03	2·90 ± 0·02	0·87	0·82	0·64	0·60
		Nuclei	8·0 ± 1·34	12·56 ± 0·01	19·73 ± 2·45	26·63 ± 3·59	4·29	3·82	4·50	5·54
		Supernatant	0	9·40	7·20 ± 1·01	1·92 ± 0·10		2·86	1·64	0·38
Plasma membrane	5′-Nucleotidase	Whole homogenate	20·26 ± 2·37 (3)	28·15 ± 2·53 (4)	69·82 ± 3·44 (3)	178·8 ± 20·13 (4)	100	100	100	100
		Microsomes	6·01 ± 0·03	5·30 ± 1·23	7·10 ± 2·34	22·3 ± 4·24	29·70	18·80	10·20	12·42
		Crude mitochondria	—	16·50 ± 2·81	39·14 ± 2·91	68·7 ± 14·70		58·58	56·00	38·30
		Nerve-ending particles	2·40 ± 0·01	3·40 ± 0·22	19·83 ± 2·14	53·0 ± 8·61	11·85	12·07	28·32	29·80
		Purified mitochondria	2·70 ± 0·02	2·13 ± 0·01	7·40 ± 0·32	5·6 ± 0·32	13·32	7·60	10·58	3·12
		Crude myelin	0·99 ± 0·01	1·41 ± 0·002	3·72 ± 0·22	14·0 ± 4·25	4·87	5·01	5·34	7·80
		Nuclei	1·52 ± 0·015	1·82 ± 0·002	4·30 ± 0·03	42·7 ± 9·22	7·51	6·47	6·16	23·80
		Supernatant	4·8 ± 1·52	7·75 ± 1·00	19·70 ± 7·40	22·8	23·65	27·55	28·20	12·70
Myelin[5,6]	Leucine aminopeptidase	Whole homogenate	30·56 (2)	33·76 (4)	33·60 (2)	36·10 ± 3·22 (4)	100	100	100	100
		Microsomes	5·09	6·72 ± 2·24	5·67	4·95 ± 2·70	16·65	19·90	16·90	13·70
		Crude mitochondria	—	17·30 ± 3·0	13·37	17·90 ± 2·14		51·42	39·91	49·52
		Nerve-ending particles	7·60	7·11 ± 1·79	4·50	5·89 ± 1·48	24·90	21·15	13·40	16·30
		Purified mitochondria	3·79	2·61 ± 1·14	3·37	3·96 ± 0·05	12·41	7·75	10·00	10·98
		Crude myelin	5·18	5·76 ± 1·34	5·60	6·20 ± 0·01	16·98	17·10	16·65	17·15
		Nuclei	5·21	1·18 ± 0·01	4·00	3·21 ± 2·54	17·09	3·50	11·90	8·90
		Supernatant	3·64	7·05 ± 3·82	6·10	11·60 ± 2·55	11·92	20·09	18·15	32·18

Myelin?	2',3'-Cyclic-nucleotide 3'-phosphohydrolase*	Whole homogenate	—	1902±348 (5)	4015 (1)	5456±372 (3)	100	100	100	100
		Microsomes	—	264±10·4	—	633±70·5		13·90		14·6
		Crude mitochondria	—	1015±39·45	—	—		55·02		
		Nerve-ending particles	—	242±34·5	—	337±48·7		12·70		6·15
		Purified mitochondria	—	89±9·8	—	—		4·70		
		Crude myelin	—	613±84·5	1811	2968±146		32·20	45·2	54·40
		Nuclei	—	246±30·9	—	—		12·9		
		Supernatant	—	250±16·5	—	—		13·1		

*It has been found that phosphohydrolase activity of all fractions may be increased three times by prior treatment with 1% (w/v) Triton X-100 in place of sonication.

Correlations of these morphological changes with the biochemistry of the individual subcellular fractions of developing brain presents certain difficulties, many of which can only partially be overcome. One special problem is that separated fractions are usually heterogeneous (Petersen & Schou, 1955; Aldridge, 1957; Petrushka & Giuditta, 1959), and this is particularly true of subcellular fractions isolated from the developing brain. Thus, although myelin when prepared from mature nervous tissue gives one of the purest fractions (Autilio, Norton & Terry, 1964), samples isolated from developing brain are quite heterogeneous (Eng & Noble, 1968). Similarly, heterogeneity is seen in other fractions, e.g. isolated synaptic-ending fractions from newborn compared to adult brain (Spence & Wolfe, 1967). However, by 12 days after birth most of the isolated subcellular fractions from rat brain appear to be more uniform, whereas the myelin fraction still remains of uncertain composition. In an attempt to unravel some of these problems we have studied the distribution of enzyme activity and the chemical composition of different subcellular fractions at various stages of late development.

Synaptic endings and mitochondria in the developing brain. In the restricted age-group examined in the present study, as was expected, little change with age was observed in the concentration of total nucleic acid in each fraction. Protein concentration in mitochondrial and synaptic-ending fractions about doubles from 12 days after birth to maturity, and apart from a substantial increase in the amount of protein in myelin little change is seen in other fractions. The alterations in synaptic-ending protein can be compared with changes in gangliosides in the developing brain, for there is evidence that these lipids are localized in the synaptic membranes. Measurement of ganglioside content of rat brain shows that this is maximal by 25 days after birth (Spence & Wolfe, 1967; Rubiolo De Maccioni & Caputto, 1968), suggesting that by then the adult population of synaptic connexions is established within the central nervous system. During the period from 12 days after birth to maturity there is a threefold increase in the total activity of acetylcholinesterase in the synaptic-ending fraction. This increase may not only be correlated with the multiplication of synapses, but it may also reflect the increased functional activity of the developing brain. Thus similar changes are seen in the increased activity of other enzymes, e.g. cholinesterase, ATPase and aryl esterase, all of which may have a role in transmission processes.

During development succinate dehydrogenase activity rapidly rises, reaching the value in the adult brain by about 25 days after birth (Davison & Gregson, 1962; Pitts & Quick, 1967). Similar changes were seen in the present study in the

Table 4. *Composition of rat brain myelin fractions and microsomes*

Methods of separation are described in the text. Lipid analyses were by the methods used by Cuzner & Davison (1967) and results are recorded as molar ratios (cholesterol = 100). The numbers of observations are shown in parentheses.

Fractions	Age	Protein (mg./g. wet wt.)	Lipid molar ratio cholesterol : phospholipid : cerebroside	Proportion of phosphoglyceride fatty acids C_{16} and smaller (%)
Crude myelin	15 days	5·15 (5)	100:140:10*	40·21 (3)
	Adult	13·40 (5)	100:95:35*	17·32 (4)
Purified myelin	15 days	1·76 (5)	100:130:37	36·25 (2)
	Adult	12·20 (3)	100:80:26*	18·84 (2)
Second myelin-like fraction	15 days	2·07 (5)	100:120:12	38·51 (2)
	Adult	1·05 (3)		36·69 (2)
Microsomes	15 days	13·40 (5)	100:158:2*	43·13 (3)
	Adult	11·90 (5)	100:120:14*	36·62 (3)

* From Cuzner & Davison (1968).

Table 5. *Fatty acids of rat brain myelin and microsomes during development*

Methods of analysis were described in the text. Results are given as a percentage of total fatty acids of chain length C_{14} to C_{20}. Numbers in parentheses indicate numbers of separate experiments.

Fatty acid	Microsomes				Crude myelin			
Age (days) ...	10 (3)	15 (3)	21 (2)	Adult (3)	12 (3)	15 (3)	21 (2)	Adult (4)
$C_{14:0}$	1·5	1·3	2·50	1·7	2·44	1·74	0·93	0·55
$C_{14:1}$	*	—	0·28	—	—	—	—	—
$C_{15:0}$	0·32	—	0·50	0·42	0·81	0·55	0·69	—
$C_{15:1}$	0·93	0·93	0·75	1·20	0·83	1·51	1·66	1·52
$C_{16:0}$	39·9	37·70	30·0	30·4	43·70	33·80	18·80	13·38
$C_{16:1}$	5·2	3·2	2·9	2·9	3·82	2·61	2·38	1·87
$C_{17:0}$	—	—	0·5	0·41	—	—	0·50	0·64
$C_{17:1}$	—	1·7	1·3	1·8	2·13	2·22	1·66	2·29
$C_{18:0}$	22·7	23·0	21·6	26·3	19·90	22·35	19·05	20·38
$C_{18:1}$	17·5	18·1	19·3	22·5	20·90	24·20	38·35	40·99
$C_{18:2}$	1·4	—	1·7	1·3	1·0	1·50	1·80	1·79
$C_{20:0}$	—	—	—	—	—	—	—	—
$C_{20:1}$	—	—	—	—	1·17	2·00	4·02	8·32
$C_{20:4}$	10·0	13·4	18·0	10·0	4·17	8·15	10·24	8·19
% C_{16} and below	47·85	43·13	36·93	36·62	51·20	40·21	24·46	17·32

* Less than 0·25% of the total fatty acids.

enzyme activity of the purified mitochondrial fraction (Table 3). The specific activity of the mitochondrial-fraction succinate dehydrogenase increases threefold in comparison with that in the isolated synaptic-ending fraction. These findings may simply be due to the greater physical stability of the synaptic endings in the adult compared with the young brain, or it may be that other explanations apply. Balázs, Kovács, Teichgräber, Cocks & Eayrs (1968) suggest that mitochondria from nerve endings have different enzyme activities from those of the free organelle, and the present observations may therefore indicate a higher rate of synthesis of succinate dehydrogenase in 'free' mitochondria compared with those in the synapse. According to Gregson & Williams (1969) there is no significant difference in the number of mitochondria in the newborn and adult rat brain (about 36×10^{10} mitochondria/g. wet wt.), but they find succinate dehydrogenase activity in mitochondria increases with maturation of the animal. Increase of 'free' mitochondrial succinate dehydrogenase activity may also be related to the fundamental changes in glucose metabolism, for during development glycolysis in the newborn brain is largely replaced by respiratory activity (Davison & Dobbing, 1968).

Table 6. *Fatty acids of purified myelin and second fraction in developing rat brain*

Isolation of purified myelin and second fraction was described in the text. Results are expressed as a percentage of total fatty acids of chain length C_{14} to C_{20}. Numbers in parentheses indicate numbers of separate experiments.

Fatty acid	Purified myelin			Second myelin-like fraction		
Age (days) ...	12 (2)	15 (2)	Adult (2)	12 (2)	15 (2)	Adult (2)
$C_{14:0}$	1·76	1·33	0·33	3·02	1·44	0·88
$C_{14:1}$	—	—	—	—	—	—
$C_{15:0}$	0·29	—	—	—	0·49	—
$C_{15:1}$	0·44	3·95	2·10	1·09	1·11	1·40
$C_{16:0}$	40·33	28·22	15·44	37·53	32·72	32·40
$C_{16:1}$	3·67	2·75	0·95	5·49	2·75	2·01
$C_{17:0}$	—	—	0·28	—	—	—
$C_{17:1}$	0·43	3·35	3·68	0·79	0·90	3·01
$C_{18:0}$	21·28	19·28	26·62	17·61	19·71	26·56
$C_{18:1}$	18·14	21·32	35·55	16·03	14·13	26·66
$C_{18:2}$	5·22	5·64	1·26	3·33	5·48	—
$C_{20:0}$	—	—	0·96	—	—	—
$C_{20:1}$	—	3·84	6·81	5·02	4·04	—
$C_{20:4}$	8·38	11·28	5·95	10·04	11·72	7·04
$C_{22:6}$*	—	—	—	—	5·48	—
%C_{16} and below	46·49	36·25	18·84	47·13	38·51	36·69

* Tentative identification

Crude myelin and the second myelin-like fraction. The present work indicates that 'crude' myelin from developing brain, like that of the adult, is generally low in enzymic activity (Adams *et al.* 1963; Miani, Cavallotti & Caniglia, 1969). Of the enzymes we have studied apart from leucine aminopeptidase and 2′,3′-cyclic nucleotide 3′-phosphohydrolase activity no other enzyme accounts for more than 10% of that in the whole brain (Tables 2 and 3). Moreover, the enzyme specific activity is always less than that of any other fraction (Table 7). These findings make it unlikely that the isolated myelin is extensively contaminated by other subcellular fractions. For example, relatively less ATPase and more leucine aminopeptidase activity is present in myelin compared with that in the microsomal fraction, and little succinate dehydrogenase activity was found (see Table 7).

However, it has been shown that 'crude' myelin isolated from developing rat brain is heterogeneous (Eng & Noble, 1968), and W. T. Norton, A. N. Davison & M. Spohn (unpublished work) have shown that crude myelin from a 15-day-old rat brain can be further separated on either sucrose or caesium chloride gradients into two fractions. One has a composition similar to mature myelin and the second fraction is comparable in composition with other cellular membranes. Thus, as we report in the present paper, the second myelin-like fraction, isolated on sucrose density gradients as described in the Experimental section, contains only traces of cerebroside, more phosphatidylcholine is present than in mature myelin and the proportion of shorter-chain saturated phosphoglyceride fatty acids is greater than is found in mature myelin. Rat liver cell membranes isolated by Skipski *et al.* (1965) contain about 35% of both $C_{16:0}$ and $C_{17:0}$ phosphoglyceride fatty acids and about 10% of $C_{18:1}$, $C_{18:2}$ and $C_{20:4}$ acids. About twice as much palmitate (28%) and stearate (31%) as linoleate (14%) and arachidonate (14%) was found in rat liver plasma membrane phosphoglyceride by Pfleger, Anderson and Snyder (1968). Both the lipid composition of the second myelin-like fraction in the immature brains and the pattern of its phosphoglyceride fatty acids is therefore different from that of mature myelin and resembles that of other cellular membranes and in particular plasma membrane. It is therefore clearly possible, from lipid and fatty acid composition alone, to distinguish between mature-type myelin and all other neural membranes. From a purely chemical standpoint, however, it is not possible to differentiate between the second myelin-like fraction and other membrane fractions, particularly those morphologically similar membranes derived from synaptic endings and microsomes. Nerve-ending 'ghosts' have a high phospholipid and low cerebroside content (Whittaker, 1966). However, useful information can be derived from examination of the enzyme profiles for the different subcellular fractions. The presence in immature brain of high acetylcholinesterase activity in synaptic endings makes it unlikely that the second membrane fraction is grossly contaminated by

Table 7. *Enzyme activity of rat brain myelin fractions*

Results are the means of analyses of several experiments (numbers shown in parentheses) and recorded for enzyme activity as μmoles of substrate utilized/hr./mg. of protein under the conditions stated above. Methods are given in the text. The enzyme specific activity (μmoles of substrate/hr./mg. of protein) and the typical localizations for each enzyme are shown.

Enzyme	15-day-old rat				Adult rat			
	Typical localization and specific activity (activity/hr./mg. of protein)	Myelin fractions; specific activity (activity/hr./mg. of protein)			Typical localization and specific activity (activity/hr./mg. of protein)	Myelin fractions; specific activity (activity/hr./mg. of protein)		
		Crude myelin	Purified myelin	Second fraction		Crude myelin	Purified myelin	Second fraction
Succinate dehydrogenase	Free mitochondria, 11·90 Nerve-ending particles, 7·00	0·54 (4)	0·88 (4)	0·43 (4)	Free mitochondria, 36·26 Nerve-ending particles, 3·31	0·21 (3)	0·15 (3)	1·1 (3)
$Na^+ + K^+ + Mg^{2+}$-effected ATPase	Microsomes, 17·4 Nerve-ending particles, 19·1	11·0 (4)	21·0 (4)	10·1 (4)	Microsomes, 33·8 Nerve-ending particle, 29·7	9·4 (3)	4·7 (3)	29·0 (3)
Acetylcholin-esterase	Microsomes, 5·7 Nerve-ending particles, 5·2	2·5 (3)	5·8 (2)	1·45 (2)	Microsomes, 10·6 Nerve-ending particles, 8·9	2·7 (3)	2·2 (2)	6·3 (2)
Cholinesterase	Microsomes, 0·28 Nerve-ending particles, 0·13	0·19 (3)			Microsomes, 0·75 Nerve-ending particles, 0·28	0·17		
Aryl esterase	Microsomes, 5·78 Nerve-ending particles, 2·29	2·9 (3)			Microsomes, 9·46 Nerve-ending particles, 3·31	1·98		
5′-Nucleotidase	Microsomes, 0·39 Supernatant, 0·33	0·29 (4)	0·33 (3)	0·20 (3)	Microsomes, 1·87 Supernatant, 1·09	1·04 (4)	0·65 (2)	0·30 (2)
Leucine aminopeptidase	Microsomes, 0·51 Nerve-ending particles, 0·32	1·20 (4)	1·37 (3)	1·27 (3)	Microsomes, 0·41 Nerve-ending particles, 0·20	0·47 (4)	0·33 (3)	0·99 (3)
2′,3′-Cyclic nucleotide 3′-phosphohydrolase	Microsomes, 19·71 Nerve-ending particles, 10·85	128 (5)	166 (5)	54·2 (5)	Microsomes, 53·05 Nerve-ending particles, 11·60	221 (5)	202 (3)	86·5 (3)

synaptic membranes. The absence of highly basic encephalitogenic protein from the myelin-like fraction (H. C. Agrawal, N. L. Banik, A. H. Bone, A. N. Davison & M. Spohn, unpublished work) makes it unlikely that it is degraded myelin, as suggested by Eng & Noble (1968). The electrophoretic pattern in phenol–acetic acid–water (2:1:1, w/v/v) (Takayama, Maclennan, Tzagoloff & Stoner, 1964) of the total protein from synaptic endings is different from that of the second myelin-like fraction, but the latter resembles the proteins of the microsomal fraction. Similar differences were observed by Cotman & Mahler (1967) between adult myelin and synaptic-ending proteins.

Enzyme activities determined in the microsomal fraction do not support the view that the myelin-like fraction is typically microsomal. Nevertheless, microsomes are a highly heterogeneous mixture of membranes (Ernster, Siekevitz & Palade, 1962; Sellinger *et al.* 1966; Reid, 1967) and it is possible that a submicrosomal fraction has similar properties to that of the second myelin-like fraction. Nor is some resemblance of this fraction to that of smooth microsomes surprising, for plasma membranes from neurones and glia as well as the membrane-synthesizing machinery of the cell are located in this microsomal subfraction.

An alternative possibility is that the second myelin-like fraction is not a contaminant but rather a hitherto undescribed membrane fraction present mainly in the developing central nervous system. It has been proposed (Davison *et al.* 1966; Banik *et al.* 1968) that such a fraction may be derived from glial plasma membrane and thus be regarded as a transitional form of membrane leading to the synthesis of myelin (see Benson, 1966). Histological observations support this hypothesis, for during myelination there are changes in the staining

properties of newly deposited myelin (Banik *et al.* 1968; Wolman, 1957), and during early stages of myelination loose whorls of membrane assemble around the axon before compact myelin is formed (De Robertis, Gerschenfeld & Wald, 1958; Caley & Maxwell, 1968). These single membranes, loosely attached to compact myelin and behaving on centrifugation as myelin may become separable after osmotic shock. Similarity between myelin and the second myelin-like fraction is seen in the presence of relatively high leucine aminopeptidase activity (Beck *et al.* 1968) and particularly 2′,3′-cyclic nucleotide 3′-phosphohydrolase activity, for the latter enzyme is regarded by Kurihara & Tsukada (1967) as being 'localized in the myelin sheath or its intimately associated structures'.

As far as we know, the morphological and biochemical characteristics of the myelin-like fraction as presented in this paper have not been described previously. It therefore appears that the new membrane fraction is either principally derived from oligodendroglial plasma membrane, or is a submicrosomal contaminant present in relatively high proportion in the developing brain. The true identity of the new membrane fraction will remain a matter of conjecture until pure glial plasma membrane is isolated from developing brain. Characterization of proteins of the new membrane fraction will help in understanding the structural differences and the functional roles of the different membrane components of the developing nervous system.

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